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Dopamine Signaling Promotes The Xenobiotic Stress Response And Protein Homeostasis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 27 August 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, the referees find the manuscript interesting. However, they also indicate that further analysis is needed to consider publication here. In particular, DA signaling needs to be better linked mechanistically with the UPS pathway and further insight into the cells that mediate dopamine receptor function in proteostasis is also needed. Should you be able to extend the analysis along these lines and to address the other concerns as well, then we would like to consider a revised version.

I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the major concerns at this stages.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this paper the authors show that dopamine and the D1-like receptors DOP-1 and DOP-4 are involved in the control of proteostasis in C. elegans epithelial tissues. They further show that pathogenic bacteria enhance proteasome activity in a largely dopamine-dependent manner and provide evidence that dopaminergic neurons sense pathogenic bacteria directly through mechanosensation to promote an epithelial stress response.

Overall this is an interesting paper describing a novel but potentially conserved role for dopamine in innate immunity and aging. In principle I think it is appropriate for the EMBO journal. A weakness of the paper is that there is almost no investigation of this phenomenon at the cellular level; in particular, the cells in which the dopamine receptors function is not addressed. Without this information, the study feels somewhat preliminary.

Specific comments:

1. I think the most important way the paper could be improved would be to establish the site of action of dop-1 and/or dop-4. Do either of these receptors act directly in intestine or hypodermis for those phenotypes, or alternatively do they function in neurons or gland cells that might release another modulator? Cell/tissue-specific rescue experiments could easily address these questions.

2. Similarly, the authors conclude from the trp-4 and mec-5 experiments that mechanosensation by the dopaminergic neurons is involved in the regulation of proteostasis by pathogenic bacteria. However, both these genes are expressed in additional neurons besides the dopaminergic cells. It is important to see if the phenotypes observed can be rescued by expression of a wild-type transgene under a dopaminergic promoter.

Minor comments

On page 8, the authors state "turnover is reduced in mec-5 and trp-4 mutants, although not to the same extent as in cat-2, dop-1 or dop-3". Is this correct, or should the sentence read "dop-4" instead of "dop-3"?

Another monoamine, serotonin, was recently shown to be involved in the control of innate immune responses in rectal epithelial cells in response to pathogenic bacteria (Anderson et al., Plos Pathogens 2013). Given the links between dopamine and serotonin signaling in worms and elsewhere, it might be worthwhile for the authors to discuss how their results might relate to these earlier findings.

Referee #2:

Review of EMBO J Manuscript Joshi et al

In this manuscript, the authors use a ubiquitin/proteasome substrate screening approach to define biological pathways involved in altering the activity of the ubiquitin proteasome system (UPS) in C. elegans epithelia. Using this approach, they identify dopaminergic signaling as a mechanism involved in altering UPS activity during work development (specifically following peak fecundity). The show that DA signaling induces xenobiotic stress-responsive genes through the activity of the dopamine receptor DOP-1, which they predict is involved in promoting UPS activity. They then demonstrate that the DA-dependent alterations in UPS are induced by pathogenic bacteria and that impairment of DA signaling sensitizes worms to bacterial infection and heat shock.

Overall, the topic of study is interesting. The deconvolution of molecular mechanisms involved in coordinating organismal proteostasis is of high interest and is important. Regardless, there are a number of major issues that need to be addressed in this manuscript. Most notably, there is no clear connection between DA signaling and the UPS system. The authors show that DA signaling does not increase proteasome subunits or proteasome activities (although this work was done in complete worm lysis and not specifically monitoring epithelial proteasome activity). Furthermore, the authors do not identify specific UPS components that are transcriptionally regulated by DA signaling. Instead, the authors rely solely on the Ub-GFP assay to define UPS activity in specific epithelia cells. The knockdown of DA signaling factors and select xenobiotic response genes does appear to influence the stability of the Ub-GFP probe, but these results alone are not sufficient to demonstrate the proposed link between altered DA signaling and altered UPS activity. While it is clear that dopamine has an important role in some aspect of proteostasis maintenance in these cells, the link with UPS remains poorly defined. Considering the topic of this manuscript, this link must be established clearly with additional experimental efforts.

I further discuss this and other major issues with the manuscript below.

1. The authors' use of the Ub-GFP assay to define UPS activity is fine for screening, but additional assays must be used to further validate the altered activity of this pathway. For example, it is not clear that the assay selectively reports on proteasome-dependent Ub-GFP degradation independent of altered expression. If the Ub-GFP substrate has a substantially shorter half-life than the RFP reporter than the use of the RFP as an expression control is compromised. Thus, the differences between Ub-GFP levels and RFP could still be explained by altered expression of these genes driven by the tissue-specific promoters. While this is a great assay for screening, in order to claim that DA signaling affects UPS activity, the authors must identify 'how' this occurs using other approaches. The authors propose that this is mediated by alterations in polyubiquitination likely through the activity of ubiquitin ligases, but this is not demonstrated or discussed. There are also odd contradictory statements in the manuscript regarding poly-ubiquitination of the endogenous proteome in DA mutants. On Page 6 Paragraph 1, the authors state that 'We also observed in these mutants an increase in the levels of endogenous ubiquitinated substrates...". On Page 6 Paragraph 2, the authors state 'Moreover, the observed decrease in poly-ubiquitinated proteins in these mutants is not consistent with decreased proteasome activity." There is no quantification for these poly-Ub blots so I'm not sure which way this goes. To me, it looks like there is an accumulation of poly-ubiquitinated endogenous substrates in mutants lacking DOP-1 or DOP-4 (Fig. 2A,B), which would indicate that there is efficient ubiquitination in these mutants and that reductions in Ub-GFP ubiquitination/degradation could simply result from increased global substrates for the UPS system. If this is the case, then it would still suggest that DA signaling influences epithelial proteostasis, but a direct link to regulating UPS activity may not be the mechanism. Consistent with this model, DA mutants show increased expression of HSR target genes, indicating an imbalance in proteostasis, which the authors accurately point out. Thus, in order to demonstrate that DA signaling regulates UPS activity there must be some direct mechanistic link between DA signaling and the UPS described using alternative approaches.

2. The authors discuss previous work using the same system that shows EGF signaling is involved in regulating UPS activity during the same timeframe (L4 + 48 h) discussed in this manuscript. There is no discussion of the interplay between EGF signaling and DA signaling. This should be discussed and preferably experiments should be included that show the relationship between these pathways on epithelial proteostasis maintenance.

3. There are no error bars on Figs. 2C and 2D. These should be included. Also, these experiments measure proteasome activity from whole worm extracts. It would be interesting to evaluate whether intestinal/hypodermis proteasome activity is specifically affected. Alternatively, the authors could ask whether the alteration in Ub-GFP levels are observed in other tissues to indicate if this is a global effect or specific to epithelial tissues.

4. Figure 3B is somewhat confusing. There is an increase in Ub-GFP levels in the hypodermis of L4 + 24 animals where specific xenobiotic stress-response genes are depleted, but there appears to be no effect at the L4 + 48 point where the reduction in Ub-GFP levels are observed elsewhere in the manuscript. This is not the case in the intestines where there does appear to be stabilization of Ub-

GFP levels for both timepoints. Does this mean that the alterations in Ub-GFP levels observed at the L4 + 48 h timepoint occur through distinct mechanisms in these different cells? Or is there another explanation for this discrepancy.

5. The link between SKN-1, DAF-16, and CRH-1 is interesting, but it is important to show that these transcription factors are directly involved in inducing specific genes (e.g., xenobiotic genes) induced by DA signaling. The RNA-seq comparisons are nice, but more direct experiments are required to demonstrate that activation of these stress-responsive transcription factors are involved in the observed stabilization of Ub-GFP. The authors reference previous work suggesting this is the case, but it would be nice to validate this in their own system.

6. Lastly, it would be nice to have more data describing the link between neuronal DA signaling and intestinal proteostasis. For example, which neurons are regulating the DA-dependent effect in the hypodermis and intestines. This is a common experiment performed in projects describing non-cell autonomous signaling and would further support the results described in this manuscript.

Minor Comments.
1. The specific neurotransmitter involved in distal activation of the HSR has been identified as serotonin (Tatum et al 2015 Curr Biol). The information describing these results on Page 3 should be updated.
2. In Fig. 1B (intestines), the overlap between the GFP and RFP fluorescence in the intestines is quite poor. This should be explained. It could reflect significantly different half-lives for these proteins that would complicate the sole use of this assay to monitor UPS activity (See Major Point #1).
3. As indicated above in Major Point #1, quantifications of the endogenous poly-Ub blots are important to support claims in the manuscript.
4. In Fig. 3C, there are a couple of genes whose reduced activity does not significantly influence Ub-GFP levels at L4 + 24, but significantly increase Ub-GFP levels at L4 + 48h (e.g., ugt-62 and cyp-34A4 among others). This is interesting and some explanation for this effect should be provided.

As indicated by my comments above, this is an interesting topic of study that merits further investigation. Most notably, additional experiments must be performed to demonstrate that the observed increase of Ub-GFP levels in DA-deficient mutants are attributed to altered activity of the UPS and not simply imbalances in global proteostasis. This is an important distinction as it would suggest that DA signaling does not necessarily directly regulates the UPS (as indicated in this manuscript), but instead regulates other aspects of proteostasis in these tissues that remain to be further defined. It is possible that this alternative mechanism is the xenobiotic stress response, but if this is the case a more direct link to proteostasis must be established in this work.

Referee #3:

The manuscript "Dopamine Signaling Regulates The Xenobiotic Stress Response And The Ubiquitin Proteasome System" by Joshi et al., use the Ub676V-GFP reporter (compared to mRFP) to screen for novel modulated of the UPS system in adulthood using RNAi knockdown. They identified several dopamine (DA) signaling mutations that reduced the levels of this reporter when at the point that this reporter is rapidly degraded (L4+48hr). Of the five DA receptors, Joshi et al., find that mutants in dop-1 and dop-4 resulted in elevated levels of the reporter, suggesting that the UPS function is impaired. To determine how DA signaling affects the reporter clearance, Joshi et al., examine the levels of the Ub676V-GFP reporter and of the proteasome (subunit RPN-11 and 20S) by western blot analysis. They find increased levels of the reporter as well endogenous Ub proteins, but not of the proteasome or rate of proteasome function (in vitro) and suggest that protein poly-ubiquitylation is supported by DA signaling. Joshi et al., then use RNA-seq to examine for transcriptional changes in dop-1 mutant vs wild type. They find that dop-1 animals have reduced expression of xenobiotic and endobiotic detoxification and metabolism. They then knockdown by RNAi different dop-1 associated genes, all of which show changes in reporter stability and suggest that these detoxification genes reduce UPS function. Since DA responds to mechano-sensory stimulus, Joshi et al., examine mechanosensation mutants and find reporter stabilization here too, suggesting the environmental cues might impact UPS function via DA signaling. To test that they

then examine pathogenic bacteria PA14 effect on reporter stabilization and survival and show that PA14 can increase the levels of the reporter and that reduced survival. Finally, they use thermo-survival and Q40 aggregation to suggest that proteostasis is modulated in dop-1 mutant.

Cell-nonautonomous regulation of proteostasis and specifically neuronal regulation of distal tissue is highly interesting and very little is known on how neuronal signals can modulate proteostasis function in other tissues. The ability to sense changes in the environment that might be associated with bacterial infection and preemptively activate detoxification enzyme to inhibit such outcome is attractive. Joshi et al., demonstrate nicely that dop-1 is required for the expression of detoxification genes and that dop-1 mutant is more susceptible to PA14 infection. However, the link to UPS regulation is very weak. There are no changes in UPS expression (including E3/E4 enzymes) in dop-1 mutants and the connection between dop-1 and UPS is supported mostly by the reporter stability. The only indication of general changes to protein degradation is poly-Ub high mobility smear that was not quantified and we have no indication whether this change is significant (See specific comment #1). Considering that RNAi of any detoxification gene tested resulted in reporter stabilization, suggest that the levels of this reporter may be responding to other changes in the cell. For example, competition for Ub enzymes with other substrates might slow down its degradation (See specific comment #2). I think that the link between the UPS and DA and specifically how DA modulates DA requires a more experimental data to merit publication.

Specific comments:
1. The authors say that the data presented in Fig. 1 indicate that "DA signaling promotes UPS activity in epithelia via activation of D1-like DARs". However, this statement is supported only by the effect of these mutants on Ub reporter levels (compared to the expression control) examined by fluorescence (Fig 1, 2C and 2D) or by western blot analysis (Fig. 2A and 2B). This raises the question of the reporter sensitivity. How strong is the effect of these mutants on the UPS system? It is possible, for example, that the mutants caused increase in protein damage that results in increased UPS load and slower degradation. Another readout of UPS is needed to test this. Specifically given that the only effect noted in these mutants (apart from examining the reporter stabilization by different assays) is that the endogenous Ub (high mobility smear) seems stronger in the mutants. While the authors claim that "mutants for DA signaling have diminished levels of protein poly-ubiquitination", there is no quantification of this data and no statistical information. If the authors can show that the general Ub of protein is significantly enhanced (percent increase in wild type vs. mutants and statistic significant) in the 5 repeats of this experiment, then the claim that "DA signaling promotes protein poly-ubiquitination" can stand. Alternatively, the authors should use another assay to examine UPS function in vivo, since examining how DA signaling promotes protein poly-ubiquitination by RNA-seq in wild type and DA mutants yielded no further support for this claim.

2. On page 5 second paragraph the authors write: "We used these GFP-based reporters to perform an RNAi screen for regulators of UPS activity and found that mutations resulting in the loss of dopamine (DA) signaling resulted in elevated UbG76V-GFP levels in both the intestine and the hypodermis." In fact they show no data from the RNAi screen, for example the effect of RNAi knockdown of DA genes, they only show data from DA mutants (Fig. 1). Either give more information on the screen, such as screen set up, number of gene tested, number of hits, percentage of DA in hits (and possibly identify of hits, although I will understand if the authors withhold this). While the screen seems unimportant for the data presented (it is only mentioned here), it raises questions in regards to the reporter specificity. Indeed, in Fig. 3B and 3C knockdown of all genes tested stabilized the Ub reporter either on L4+24, L4+48h or both. Did these genes come up in the screen? How sensitive is the reporter to gene knockdown?

Minor comment:
1. The authors show that in wild type animals there is a difference between L4+24 and L4+48. I am guessing that the screen was conducted on L4+48. However, some genes tested affect L4+24, L4+48 or both. This is only referred to for wild type Fig. 1B-1E no information for the DA mutants on L4+24 (one mutant is shown in Fig. 3A). There is no discussion of this point for example in Fig. 3C, there is no discussion on why some genes affect only one time and others both and if this has not significance then why both are examined?

2. The inverse correlation between the reporter degradation and UPS activity makes the text hard to follow. The authors should take care in the text to help the reader when possible.

3. In Fig 1D-!E the genotype is missing in the Fig and legend.

4. On page 8 second paragraph reads: "We found that UbG76VGFP
levels relative to mRNA control". Should be mRFP?

5. There is no correlation between protein aggregation and toxicity. Some genetic modification induces aggregation, increasing toxicity and some reduced toxicity. Thus, the authors cannot conclude that decrease in aggregation they noted in Fig. 4F is due to reduced proteostasis capacity and not improved clearance of aggregates. Aggregates toxicity can indicate if proteostasis is indeed reduced or enhanced. Authors may also use other folding reporters, for the impact of DA signaling on proteostasis.

1st Revision - authors' response 23 March 2016

Reviewer #1

1. I think the most important way the paper could be improved would be to establish the site of action of dop-1 and/or dop-4. Do either of these receptors act directly in intestine or hypodermis for those phenotypes, or alternatively do they function in neurons or gland cells that might release another modulator? Cell/tissue-specific rescue experiments could easily address these questions.
We have gone back to one of the near full length GFP reporter constructs generated to examine DOP-1 expression (Tsalik et al, 2003). We observed GFP fluorescence in intestine and hypodermis, albeit at lower levels that those detected in neurons. To directly address function, we generated a transgene that expresses wild-type *dop-1* under the control of the *vha-6* promoter, the expression of which is restricted to intestine. We introduced this transgene into *dop-1* mutants that also contain the $P_{sur\text{-}5}$*::Ub*^{G76V}*-GFP* reporter and found that this rescuing transgene restored Ub^{G76V}-GFP protein turnover in *dop-1* mutants to wild-type levels. These results suggest that DOP-1 acts cell autonomously in epithelial cells to regulate proteostasis. We have included this data in Figure EV1.

2. Similarly, the authors conclude from the trp-4 and mec-5 experiments that mechanosensation by the dopaminergic neurons is involved in the regulation of proteostasis by pathogenic bacteria. However, both these genes are expressed in additional neurons besides the dopaminergic cells. It is important to see if the phenotypes observed can be rescued by expression of a wild-type transgene under a dopaminergic promoter.
We have addressed this point using a transgenic strain generated by Dr. Maria Doitsidou in her studies of the TRP-4 channel. The reviewer is correct regarding the expression of TRP-4: it is expressed in the three dopaminergic neurons (ADE, CEP, and PDE), but it is also expressed in two non-dopamineric neurons (DVA and DVC). Dr. Doitsidou created a dominant gain of function mutant TRP channel that triggers neurodegeneration in neurons (Nagarajan et al, 2014). She generated a transgene, *norSci1[*$P_{dat\text{-}1}$*::trp-4(d), unc-119(+)]*, that expresses this mutant TRP channel under the *dat-1* (dopamine reuptake pump) promoter. The $P_{dat\text{-}1}$*::trp-4(d)* only expresses the channel in the three dopaminergic neurons, and its expression results in the neurodegeneration of these cells (and not other cells like DVA and DVC). We have introduced the *Ub*^{G76V}*-GFP* reporter into a strain that expresses $P_{dat\text{-}1}$*::trp-4(d)* and found that $P_{dat\text{-}1}$*::trp-4(d)* triggers Ub^{G76V}-GFP protein stabilization. The magnitude of the effect is greater than that observed in *trp-4* mutants, but not as extensive as that observed in *cat-2* or *dop-1* mutants, which are defective for dopamine synthesis. The simplest explanation for why $P_{dat\text{-}1}$*::trp-4(d)* does not stabilize Ub^{G76V}-GFP to the same extent as do *cat-2* or *dop-1* mutations is that $P_{dat\text{-}1}$*::trp-4(d)* does not kill dopaminergic neurons in every animal. CEP neurons are killed in about 95% of animals with $P_{dat\text{-}1}$*::trp-4(d)*. By contrast, ADE and PDE are killed in only about 50% of animals with the same transgene. Thus, animals harboring the $P_{dat\text{-}1}$*::trp-4(d)* transgene will vary in phenotypic strength and would not be expected to give as strong a phenotype as observed in *cat-2* or *dop-1* knockout mutants. Regardless of this partial phenotype, other neurons that express TRP-4 (i.e., DVA and DVC) are not killed. Our results indicate that the dopaminergic are required to regulate Ub^{G76V}-GFP protein stability in epithelial tissues. We have added this data to Figure 4A.

3. Minor Comment: On page 8, the authors state "turnover is reduced in mec-5 and trp-4 mutants, although not to the same extent as in cat-2, dop-1 or dop-3". Is this correct, or should the sentence read "dop-4" instead of "dop-3"?
We apologize for this error – yes it was suppose to be *dop-4*. We have adjusted the text accordingly.

4. Minor Comment: Another monoamine, serotonin, was recently shown to be involved in the control of innate immune responses in rectal epithelial cells in response to pathogenic bacteria (Anderson et al., Plos Pathogens 2013). Given the links between dopamine and serotonin signaling in worms and elsewhere, it might be worthwhile for the authors to discuss how their results might relate to these earlier findings.
This is an excellent point, and we apologize for not including this reference in the original draft. We have added it to the Discussion section. Mechanistically, serotonin appears to work in the opposite direction and with a distinct mechanism from that of dopamine. That is, serotonin appears to be released from chemosensory neurons in response to food, and then acts on distal rectal epithelia to inhibit the immune response. It will require additional experiments beyond the scope of this paper to determine how these pathways interact.

Reviewer #2

1. The authors' use of the Ub-GFP assay to define UPS activity is fine for screening, but additional assays must be used to further validate the altered activity of this pathway. For example, it is not clear that the assay selectively reports on proteasome-dependent Ub-GFP degradation independent of altered expression. If the Ub-GFP substrate has a substantially shorter half-life than the RFP reporter than the use of the RFP as an expression control is compromised. Thus, the differences between Ub-GFP levels and RFP could still be explained by altered expression of these genes driven by the tissue-specific promoters. While this is a great assay for screening, in order to claim that DA signaling affects UPS activity, the authors must identify 'how' this occurs using other approaches. The authors propose that this is mediated by alterations in polyubiquitination likely through the activity of ubiquitin ligases, but this is not demonstrated or discussed. There are also odd contradictory statements in the manuscript regarding poly-ubiquitination of the endogenous proteome in DA mutants. On Page 6 Paragraph 1, the authors state that 'We also observed in these mutants an increase in the levels of endogenous ubiquitinated substrates...". On Page 6 Paragraph 2, the authors state 'Moreover, the observed decrease in poly-ubiquitinated proteins in these mutants is not consistent with decreased proteasome activity." There is no quantification for these poly-Ub blots so I'm not sure which way this goes. To me, it looks like there is an accumulation of poly-ubiquitinated endogenous substrates in mutants lacking DOP-1 or DOP-4 (Fig. 2A,B), which would indicate that there is efficient ubiquitination in these mutants and that reductions in Ub-GFP ubiquitination/degradation could simply result from increased global substrates for the UPS system. If this is the case, then it would still suggest that DA signaling influences epithelial proteostasis, but a direct link to regulating UPS activity may not be the mechanism. Consistent with this model, DA mutants show increased expression of HSR target genes, indicating an imbalance in proteostasis, which the authors accurately point out. Thus, in order to demonstrate that DA signaling regulates UPS activity there must be some direct mechanistic link between DA signaling and the UPS described using alternative approaches.

We have carefully analyzed both Ub^{G76V}-GFP and mRFP expression at the mRNA level in wild type and *dop-1* mutants. We have not observed a difference, and this data has been included as part of Figure 3A.

The turnover of Ub^{G76V}-GFP and other ubiquitin fusion reporters by poly-ubiquitination has been extensively studied for 30 years (Bachmair et al, 1986; Dantuma et al, 2000; Hamer et al, 2010; Koegl et al, 1999; Kuhlbrodt et al, 2011; Lindsten et al, 2003; Segref & Hoppe, 2012; Segref et al, 2011; Stack et al, 2000). For the specific transgenes used in our paper, we have validated the one expressed in the hypodermis by generating mutations in the ubiquitin residues that impair additional poly-ubiquitination. We found that mutations in K29 and K48 in which both lysine residues were converted to the non-ubiquitinatable amino acid arginine blocked Ub^{G76V}-GFP turnover and restored it to a protein expression profile over time that matched mRFP (Liu et al, 2011). Similar experiments have been performed for Ub^{G76V}-GFP under the *sur-5* promoter and Ub^{G76V}-Dendra2 in muscle (Hamer et al, 2010; Kuhlbrodt et al, 2011; Segref & Hoppe, 2012; Segref et al, 2011). In

addition, we previously showed that RNAi knockdown of proteasome subunits or mutations in E4 poly-ubiquitination enzymes stabilize the Ub^{G76V}-GFP reporter, further supporting that Ub^{G76V}-GFP turnover in wild-type animals occurs through poly-ubiquitination followed by proteolysis. We have repeated this analysis for proteasome subunit and E4 poly-ubiquitination complex RNAi knockdown and found the same result, which we have added to Figure 1B. In the case of proteasome subunit knockdown, a direct probing of GFP by Western blot shows the accumulation of stabilized, ubiquitinated Ub^{G76V}-GFP, which we have added as Extended Figure EV2. These results directly demonstrate that Ub^{G76V}-GFP undergoes poly-ubiquitination followed by proteasome-dependent degradation.

To assess the ubiquitination of the Ub^{G76V}-GFP reporter directly and specifically, it is best to examine the anti-GFP Western, which only detects Ub^{G76V}-GFP and not other endogenously ubiquitinated proteins. In wild-type animals, anti-GFP antibodies can detect a faint signal for non-ubiquitinated Ub^{G76V}-GFP and mono-ubiquitinated Ub^{G76V}-GFP (Figure 2A,B). Most Ub^{G76V}-GFP in wild type is rapidly ubiquitinated and degraded by the proteasome. By contrast, anti-GFP antibodies detect stabilized and abundant Ub^{G76V}-GFP in DA signaling mutants, most of which is in the non-ubiquitinated, mono-ubiquitinated, and di-ubiquitinated form (and a little bit of tri-ubiquitinated). This is an important point because most proteins are not formally recognized by the proteasome until they contain 4 or more ubiquitin moieties (in the case of the Ub^{G76V}-GFP chimeric protein, this would be the addition of three ubiquitin moieties because the genetically encoded Ub^{G76V} counts as the initial mono-ubiquitinated form) (Thrower et al, 2000). Ub^{G76V}-GFP would be expected to accumulate if either poly-ubiquitination or proteolysis by the proteasome were depressed. However, if depressed poly-ubiquitination were the cause, then we should see a shift of the accumulated Ub^{G76V}-GFP towards its un-ubiquitinated, mono-ubiquitinated, and di-ubiquitinated form; indeed, this is what we actually observed in DA signaling mutants. By contrast, if depressed proteolysis by the proteasome were the cause, then we should see a shift of the accumulated Ub^{G76V}-GFP towards higher mobility species indicative of the poly-ubiquitinated (i.e., >4 ubiquitin moieties) form of the reporter; however, we did not see such a shift in DA signaling mutants on the anti-GFP blot. We can at least say for Ub^{G76V}-GFP that the problem in DA signaling mutants is reduced levels of ubiquitination rather than reduced proteolysis by the proteasome.

To assess other proteins that are ubiquitinated in addition to the Ub^{G76V}-GFP, it is best to examine the anti-ubiquitin Western, which detects Ub^{G76V}-GFP, its ubiquitinated forms, and ubiquitinated forms of endogenous proteins. Because the size of the various endogenous proteins varies in the overall population of these molecules, there is no specific size mobility indicative of higher order poly-ubiquitinated proteins (compared to non-ubiquitinated, mono-ubiquitinated, and di-ubiquitinated proteins) on the gel on which we can focus. As noted by the reviewer, we do detect an increase in the multiple bands in the 50-80 kDa range in DA signaling mutants relative to wild type, suggesting that other endogenous proteins are accumulating as well. However, we have no way of knowing whether these are mono-, di-, or poly-ubiquitinated. Thus, unlike for the Ub^{G76V}-GFP reporter on the anti-GFP blot, we cannot definitively determine from this experiment if these other ubiquitinated proteins are accumulating due to depressed poly-ubiquitination or depressed proteolysis by the proteasome (or a combination of the two).

Our working model is that poly-ubiquitination but not degradation by the proteasome is depressed in DA signaling mutants. If this is true, then we should see an additive phenotype with respect to protein turnover if we examine DA signaling mutants that are also knocked down for proteasome activity. We did this experiment (as described a few paragraphs earlier) and found an additive effect in not only the accumulation of non-ubiquitinated, mono-ubiquitinated, and di-ubiquitinated Ub^{G76V}-GFP (as detected by anti-GFP Western) but also an accumulation of total endogenous ubiquitinated protein (as detected by anti-ubiquitin Western). These results are consistent with our direct proteasome activity measurements: there is plenty of proteasome capacity in DA signaling mutants, and that even with reduced ubiquitination of proteins in these mutants, much of the protein is still ubiquitinated enough to be turned over by the proteasome. We have included this data in Figure EV2.

We agree with the reviewer that an alternative explanation for why we are seeing less Ub^{G76V}-GFP turnover in DA signaling mutants is that the cells in DA signaling mutant have an increased burden of global UPS substrates. As the reviewer has highlighted, the increased expression of heat shock genes in DA signaling mutants would support this model. Consistent with this alternative model,

our results point to the upregulation of the xenobiotic response genes by DA signaling as the mechanism, protecting proteins from damage by removing toxins and ROS.

To explore this model further, we have examined several mutants that promote xenobiotic expression independent from any role in DA signaling. As observed in DA signaling mutants, mutants for xenobiotic response transcriptional regulators show Ub^{G76V}-GFP protein stabilization (they are also more sensitive to heat shock stress and are less able to sequester Poly-Q-GFP into aggregates). RNAi of many of the individual UGT and CYP genes that we found to be regulated by DA signaling also result in increased heat shock sensitivity. Moreover, we can trigger some Ub^{G76V}-GFP stabilization by treating nematodes with the ROS-generating agent paraquat. When we do the similar experiment in DA signaling mutants, we find that these mutants show much more stabilization of Ub^{G76V}-GFP than observed in wild-type animals. Similarly, mutants for heat shock chaperones, which have higher burdens of unfolded proteins, show stabilized Ub^{G76V}-GFP. Taken together, these results indicate that (1) Ub^{G76V}-GFP turnover can be blocked by extrinsic toxins like paraquat or defects in heat shock chaperones, suggesting that Ub^{G76V}-GFP stabilization is a marker for impaired proteostasis, and (2) DA signaling is required, most likely through activation of the xenobiotic response, to offset much of this damage to proteostasis by toxins like paraquat. This data has been added in Figure EV1, Figure 3D, Figure 3F, Figure 3G, and Figure 4D. We have adjusted the focus of the paper around this new hypothesis.

2. The authors discuss previous work using the same system that shows EGF signaling is involved in regulating UPS activity during the same timeframe (L4 + 48 h) discussed in this manuscript. There is no discussion of the interplay between EGF signaling and DA signaling. This should be discussed and preferably experiments should be included that show the relationship between these pathways on epithelial proteostasis maintenance.
We attempted to test this by generating double mutants between genes in the two signaling pathways. We quickly learned that there are genetic interactions between the pathways that suggest that these pathways might operate in parallel to regulate proteostasis. One of the best double mutants to analyze would have been a combination of the *dop-1* loss of function mutation, which causes decreased protein turnover, and a *let-23* gain of function mutation, which causes accelerated protein turnover. However, we found that double mutants between these two mutated genes resulted in lethality, precluding analysis. We have included a discussion of this in the Discussion section.

3. There are no error bars on Figs. 2C and 2D. These should be included. Also, these experiments measure proteasome activity from whole worm extracts. It would be interesting to evaluate whether intestinal/hypodermis proteasome activity is specifically affected. Alternatively, the authors could ask whether the alteration in Ub-GFP levels are observed in other tissues to indicate if this is a global effect or specific to epithelial tissues.
We have repeated the indicated experiments and added them, along with error bars, to Figure 2.

We suspect that the effect is primarily in epithelial tissues (hypodermis and intestine), which make up the bulk of the biomass of the adult animal. Because of germline silencing, we have not been able to assess Ub^{G76V}-GFP turnover in the germline, the other major bulk contributor to worm biomass in the adult. Nevertheless, the *$P_{sur\text{-}5}$::Ub^{G76V}-GFP* transgene can be particularly instructive in addressing this concern. While our microscopy measurements of fluorescence focus on intestine in these transgenic animals, the total lysates of these transgenics are being assayed for all tissues (the *sur-5* promoter is expressed in all tissues). Importantly, we can still see a clear, DA-signaling-dependent change in Ub^{G76V}-GFP levels in these transgenics (Figure 2B), suggesting that either this proteostasis regulation occurs in all tissues or that it is at least occurring in the tissues that, like the epithelia, contribute to the bulk mass of adults homogenized in the lysate. Given that we observe these changes in the lysate of the whole organism, if they were due to changes in proteasome activity then we should have seen such changes in our proteasome activity assays from those same lysates if such changes were actually occurring.

Ideally we would love to have an accurate, genetically encoded (so it could measure activity in specific tissues), and direct reporter for proteasome activity that did not rely on ubiquitination as a precursor step (as does the Ub^{G76V}-GFP reporter protein). To our knowledge no such reporter yet exists.

4. Figure 3B is somewhat confusing. There is an increase in Ub-GFP levels in the hypodermis of L4 + 24 animals where specific xenobiotic stress-response genes are depleted, but there appears to be no effect at the L4 + 48 point where the reduction in Ub-GFP levels are observed elsewhere in the manuscript. This is not the case in the intestines where there does appear to be stabilization of Ub-GFP levels for both timepoints. Does this mean that the alterations in Ub-GFP levels observed at the L4 + 48 h timepoint occur through distinct mechanisms in these different cells? Or is there another explanation for this discrepancy.

Generally we have found that impairment of protein ubiquitination and proteolysis by the proteasome results in two phenotypes: (1) elevation of Ub^{G76V}-GFP protein levels at all time points, and (2) a right-ward shift (i.e., delay) of the decay curve of Ub^{G76V}-GFP over time indicative of a slower rate of turnover. We have included a graph of these kinetics in Figure 1, where we show that knockdown of E4 poly-ubiquitination enzymes, proteasome subunits, and DA signaling molecules all result in elevated levels of Ub^{G76V}-GFP at nearly all time points (including L4+24 hours) and a right-ward shift of the Ub^{G76V}-GFP decay curve over time.

RNAi of *cyp-25A1*, *cyp34-A4*, and *cyp-34-A7* clearly result in Ub^{G76V}-GFP stabilization at the L4+24 hour time point. However, as the reviewer points out, it makes more sense to address the L4+48 hour time point because most Ub^{G76V}-GFP has been degraded in wild type at this time whereas Ub^{G76V}-GFP levels have stabilized in DA signaling mutants. We therefore performed an additional analysis of the L4+48 time point. We found that knockdown of *cyp-25A1*, *cyp34-A4*, and *cyp-34A7* all result in stabilization of Ub^{G76V}-GFP protein at this time point. We have added this data to Figure 3.

We expect that knockdown of any one UGT or CYP gene might give a variable result relative to knockdown of DA signaling mutants. There are many reasons for this. Different xenobiotic stress resistance genes (i.e., UGT and CYP genes) show variable expression in different tissues. Our RNA-seq analysis was performed at L4+48 hours; however, it is possible that these enzymes also show variable expression over developmental time. Both of these factors could influence the observed phenotype in tissues and time points assayed for individual RNAi knockdown experiments for each gene. Of course there is also the variable nature from gene to gene of the RNAi knockdown method as well to consider. Finally, multiple UGT and CYP genes show reduced levels (with varying levels of reduction depending on the gene) of expression in *dop-1* mutants; there is no way that RNAi can be tailored to give the exact level of gene expression reduction for any one UGT or CYP gene so that it matches its level of reduction in the *dop-1* mutant background. It is therefore expected and reasonable that knockdown experiments of individual UGT and CYP genes would give phenotypes that only partially approximate that of *dop-1* mutants; the phenotype of *dop-1* mutants likely reflects the specific changes of all of the UGT and CYP genes in aggregate.

Indeed, our RNAi data suggest that no one single UGT or CYP gene is responsible for maintaining normal levels of Ub^{G76V}-GFP turnover. We therefore decided to examine three additional genes encoding transcriptional regulators of most of the UGT and CYP genes, in aggregate, identified in our RNA-seq analysis of *dop-1* mutants: *elt-3*, *nhr-28*, and *pqm-1* (Araya et al, 2014; Budovskaya et al, 2008; Gerstein et al, 2010; Miyabayashi et al, 1999; Tepper et al, 2013). Since these three genes are being analyzed via true mutations rather than RNAi, experimental variation due to RNAi efficacy is eliminated. We found that loss of function mutations in all three of these genes result in Ub^{G76V}-GFP protein stabilization at both L4+24 and L4+48 hours. Mutants for any of these genes are more sensitive to heat shock than wild type, and mutations in *elt-3* and *nhr-28* (the two genes that we could examine) show a slower rate of $Poly(Q)_{44}$::YFP disposal into aggregates. These data have been added to Figure 3. These results, combined with the likely explanation for the variation in UGT and CYP RNAi effects described above, suggest that the overall mechanism is the same from tissue to tissue, but that the individual UGT/CYP components and their specific contribution to proteostasis varies from tissue to tissue and along different development time points.

5. The link between SKN-1, DAF-16, and CRH-1 is interesting, but it is important to show that these transcription factors are directly involved in inducing specific genes (e.g., xenobiotic genes) induced by DA signaling. The RNA-seq comparisons are nice, but more direct experiments are required to demonstrate that activation of these stress-responsive transcription factors are involved in the observed stabilization of Ub-GFP. The authors reference previous work suggesting this is the case, but it would be nice to validate this in their own system.

The gene expression profiles of *skn-1*, *daf-16*, and *crh-1* mutants are fairly well established (An & Blackwell, 2003; Mair et al, 2011; Murphy et al, 2003; Oliveira et al, 2009; Park et al, 2009). To extend our studies, we examined three transcription factors, including the conserved GATA-type regulator ELT-3, for their role in proteostasis (Araya et al, 2014; Budovskaya et al, 2008; Gerstein et al, 2010; Miyabayashi et al, 1999; Tepper et al, 2013). As described in answers to previous questions, we observed that loss of function mutations for these transcription factors yield the same defects in proteostasis as those observed in DA signaling mutants. We have noted that ChIP-seq analysis identified these transcription factors, as well as SKN-1, DAF-16, and CRH-1, as binding near almost all of the UGT and CYP genes identified in our analysis. Given the well-established role of CREB (CRH-1 and CRH-2 in *C. elegans*) as a transcriptional output for dopaminergic signaling (Beaulieu & Gainetdinov, 2011; Cadet et al, 2010; Mair et al, 2011; Suo & Ishiura, 2013), we suspect that it is the transcription factor through which dopaminergic signaling manifests its effect on the expression of UGT and CYP genes. Most likely SKN-1, DAF-16, ELT-3, NHR-28, and PQM-1 help promote UGT and CYP gene expression. A complete demonstration of this hypothesis will require the generation of low-copy tagged versions of each of these transcription factors followed by extensive ChIP-seq experiments to determine which of these factors demonstrates dopamine-dependent binding to UGT and CYP promoter elements. We feel that such analysis goes beyond the scope of this manuscript.

6. Lastly, it would be nice to have more data describing the link between neuronal DA signaling and intestinal proteostasis. For example, which neurons are regulating the DA-dependent effect in the hypodermis and intestines. This is a common experiment performed in projects describing non-cell autonomous signaling and would further support the results described in this manuscript.
As discussed for the comment made by reviewer 1, we have addressed this point by using the published $P_{dat\text{-}1}$*::trp-4(d)* transgene to directly kill just the three dopaminergic neurons (CEP, ADE, and PDE). Neurodegeneration of these neurons alone is sufficient to cause Ub^{G76V}-GFP stabilization. These neurons release dopamine from sensory endings into the pseudocoelomic body cavity, bathing epithelial tissues and allowing DA to act in a neurohormonal fashion. Using a GFP reporter, we find that DOP-1 is expressed in hypodermal and intestinal tissues. As discussed in our comments to reviewer 1, we have directly shown that DOP-1 is required cell autonomously in the case of the intestine. The simplest model to account for our observations is one in which the dopaminergic neurons release DA into the body cavity, where it diffuses and binds to DA receptors on intestinal and hypodermal epithelia.

Minor Comments:

1. The specific neurotransmitter involved in distal activation of the HSR has been identified as serotonin (Tatum et al 2015 Curr Biol). The information describing these results on Page 3 should be updated.
We apologize for this oversight. We have added this information and the reference to the indicated section.

2. In Fig. 1B (intestines), the overlap between the GFP and RFP fluorescence in the intestines is quite poor. This should be explained. It could reflect significantly different half-lives for these proteins that would complicate the sole use of this assay to monitor UPS activity (See Major Point #1).
The integrated reporter used is comprised of two transgenes: $P_{col\text{-}19}$*::*Ub^{G76V}*-GFP* and $P_{col\text{-}19}$*::mRFP*. In early stages of development (e.g., L4+24h), the levels and expression pattern of these two proteins is similar. As animals begin to enter peak fecundity, we observe a rapid turnover of Ub^{G76V}-GFP protein but not mRFP. Thus, in animals that are L4+48 hours old and onward, we expect to see no overlap between Ub^{G76V}-GFP and mRFP signals because the Ub^{G76V}-GFP has undergone turnover whereas the mRFP (an internal control for differences in expression from the shared *col-19* promoter) remains stable. In *C. elegans*, GFP and mRFP show similar expression and turnover, as we previously showed that either GFP alone or Ub^{G76V}-GFP with mutations in the key lysines in the ubiquitin moiety show a similar pattern of expression and turnover as does the mRFP control (Liu et al, 2011). Finally, knockdown of the proteasome subunits stabilizes Ub^{G76V}-GFP, again yielding a similar pattern of expression and turnover to that observed for mRFP. The half-lives of GFP and mRFP are quite similar in *C. elegans*; the addition of the Ub^{G76V} to GFP

dramatically shortens its half-life relative to that of mRFP in an ubiquitin- and proteasome-dependent manner.

3. As indicated above in Major Point #1, quantifications of the endogenous poly-Ub blots are important to support claims in the manuscript.
We have carefully repeated our analysis of wild type versus *dop-1* mutants using anti-ubiquitin antibodies, quantifying the endogenous ubiquitinated proteins in the 50-80 kDa range. We have added this data to Figure EV2.

4. In Fig. 3C, there are a couple of genes whose reduced activity does not significantly influence Ub-GFP levels at L4 + 24, but significantly increase Ub-GFP levels at L4 + 48h (e.g., ugt-62 and cyp-34A4 among others). This is interesting and some explanation for this effect should be provided.
As discussed several paragraphs earlier, there are multiple reasons why RNAi knockdown of any one UGT or CYP gene would only display partial phenotypes relative to that of *dop-1* mutants. These reasons include differences in CYP and UGT gene expression in tissues and developmental timing, differences in RNAi efficacy, differences in the contribution of any given CYP or UGT gene to the aggregate phenotype, and differences in the level of mRNA reduction of these genes in the *dop-1* mutant relative to wild type.

Reviewer #3

1. The authors say that the data presented in Fig. 1 indicate that "DA signaling promotes UPS activity in epithelia via activation of D1-like DARs". However, this statement is supported only by the effect of these mutants on Ub reporter levels (compared to the expression control) examined by fluorescence (Fig 1, 2C and 2D) or by western blot analysis (Fig. 2A and 2B). This raises the question of the reporter sensitivity. How strong is the effect of these mutants on the UPS system? It is possible, for example, that the mutants caused increase in protein damage that results in increased UPS load and slower degradation. Another readout of UPS is needed to test this. Specifically given that the only effect noted in these mutants (apart from examining the reporter stabilization by different assays) is that the endogenous Ub (high mobility smear) seems stronger in the mutants. While the authors claim that "mutants for DA signaling have diminished levels of protein poly-ubiquitination",
there is no quantification of this data and no statistical information. If the authors can show that the general Ub of protein is significantly enhanced (percent increase in wild type vs. mutants and statistic significant) in the 5 repeats of this experiment, then the claim that "DA signaling promotes protein poly-ubiquitination" can stand. Alternatively, the authors should use another assay to examine UPS function in vivo, since examining how DA signaling promotes protein poly-ubiquitination by RNA-seq in wild type and DA mutants yielded no further support for this claim.
As mentioned previously, we have carefully repeated our analysis of wild type versus *dop-1* mutants using anti-ubiquitin antibodies, quantifying the endogenous ubiquitinated proteins in the 50-80 kDa range. We have added this data to Figure EV2.

2. On page 5 second paragraph the authors write: "We used these GFP-based reporters to perform an RNAi screen for regulators of UPS activity and found that mutations resulting in the loss of dopamine (DA) signaling resulted in elevated UbG76V-GFP levels in both the intestine and the hypodermis." In fact they show no data from the RNAi screen, for example the effect of RNAi knockdown of DA genes, they only show data from DA mutants (Fig. 1). Either give more information on the screen, such as screen set up, number of gene tested, number of hits, percentage of DA in hits (and possibly identify of hits, although I will understand if the authors withhold this). While the screen seems unimportant for the data presented (it is only mentioned here), it raises questions in regards to the reporter specificity. Indeed, in Fig. 3B and 3C knockdown of all genes tested stabilized the Ub reporter either on L4+24, L4+48h or both. Did these genes come up in the screen? How sensitive is the
reporter to gene knockdown?
We have included additional information on the screen in the Results section and the Methods section. This was an RNAi screen performed in a non-sensitized background. Animals were raised on RNAi bacteria from embryo stage through until L4+48 hours, at which point they were scored for any stabilization of Ub^{G76V}-GFP relative to mRFP control. The RNAi collection used was that generated by the lab of Josh Kaplan (Sieburth et al, 2005). It represents only 2072 genes out of the

total genome, but it is enriched for signal transduction molecules, cytoskeletal regulators, and membrane trafficking molecules. Out of these 2072 genes, we identified positives for 31 candidates, including for one of the dopamine receptors. RNAi screens can give false positives and variable results; therefore, we analyzed the remaining DA signaling genes using true mutants to avoid possible variability due to the RNAi method. Several of the positive candidates encode components of the EGFR signal transduction pathway that we previously showed regulates Ub^{G76V}-GFP turnover (Liu et al, 2011). We are analyzing the remaining candidates and plan to publish them separately. It should be noted that *cat-2*, *pbs-4*, *pbs-5*, *rpn-11*, *ufd-1*, *daf-16*, *skn-1*, *elt-3*, *crh-1*, *crh-2*, *pqm-1*, and all of the UGT and CYP genes examined were not represented in the RNAi library; thus, we could never have identified these genes in our screen.

Minor Comments:

1. The authors show that in wild type animals there is a difference between L4+24 and L4+48. I am guessing that the screen was conducted on L4+48. However, some genes tested affect L4+24, L4+48 or both. This is only referred to for wild type Fig. 1B-1E no information for the DA mutants on L4+24 (one mutant is shown in Fig. 3A). There is no discussion of this point for example in Fig. 3C, there is no discussion on why some genes affect only one time and others both and if this has not significance then why both are examined?
As discussed in the response to the other two reviewers, a decrease in Ub^{G76V}-GFP turnover can be observed as (1) an increase in the steady state level of the reporter protein at any of the time points and (2) a rightward shift in the turnover curve of that protein with respect to time. This can be observed when animals are exposed to a chronic RNAi knockdown of proteasome subunits (Figure 1B), and we observed both an increase in Ub^{G76V}-GFP steady state levels at all time points (including L4+24 hours) and a rightward shift in the turnover curve in DA signaling mutants like *cat-2* and *dop-1* (Figure 1K). Our results suggest that DA signaling is continually promoting Ub^{G76V}-GFP turnover at all of the time points assayed, including L4+24 hours (rather than just triggering the increase in turnover observed at L4+48 hours and beyond). We hypothesize that DA signaling performs this function by promoting the expression of multiple xenobiotic detoxification genes (i.e., the UGT and CYP genes). Since DA signaling is activating the expression of multiple such genes, it is reasonable to expect that knockdown by RNAi of any one gene is unlikely to cause a phenotype that is equivalent in tissue affected, timing, and strength to the *dop-1* or *cat-2* loss of function mutants. In interpreting RNAi knockdown experiments of single UGT and CYP genes, one must also consider differences in CYP and UGT gene expression in tissues and developmental timing, differences in RNAi efficacy, differences in the contribution of any given CYP or UGT gene to the aggregate phenotype, and differences in the level of mRNA reduction of these genes in the *dop-1* mutant relative to wild type. We have added a brief explanation of this point in the Results section. Importantly, we have also analyzed mutants for three regulators (*elt-3*, *nhr-28*, and *pqm-1*) of aggregate CYP/UGT expression so as to more closely parallel the changes in aggregate CYP/UGT expression observed in the *dop-1* mutants. Our results with mutants from these genes show phenotypes that are consistent with *dop-1* mutants. We have added this data to Figures 3 and 4. We have also restricted most of our analysis to the L4+48 hour time point to avoid potential confusion.

2. The inverse correlation between the reporter degradation and UPS activity makes the text hard to follow. The authors should take care in the text to help the reader when possible.
We have adjusted the wording to focus on Ub^{G76V}-GFP turnover rather than UPS activity, which should make it easier for readers to follow the figures and data. The cartoon in Figure 1A should also help explain the reporter assay. Finally, we have expanded and simplified the model figure into two components (Figure 5 and Figure EV4).

3. In Fig 1D-1E the genotype is missing in the Fig and legend.
We apologize for the lack of clarification on these figures. These original figures were from wild-type animals. The idea behind these figures was to show the change in Ub^{G76V}-GFP levels over time. We have created new time course graphs with more time points, which we have substituted for these two figures. These new graphs are Figure 1B and Figure 1K. Genotypes are clearly labeled on the new graphs.

4. On page 8 second paragraph reads: "We found that UbG76VGFP levels relative to mRNA control". Should be mRFP?

Our apologies – yes it should be mRFP. The two words look so alike that we missed it during our editing. We have made the appropriate change.

5. There is no correlation between protein aggregation and toxicity. Some genetic modification induces aggregation, increasing toxicity and some reduced toxicity. Thus, the authors cannot conclude that decrease in aggregation they noted in Fig. 4F is due to reduced proteostasis capacity and not improved clearance of aggregates. Aggregates toxicity can indicate if proteostasis is indeed reduced or enhanced. Authors may also use other folding reporters, for the impact of DA signaling on proteostasis.

We agree that there is no correlation between protein aggregation and toxicity. Indeed, failure to sequester unfolded and/or damaged proteins into aggregates is likely to be more toxic for cells than carrying around the aggregates themselves. In *C. elegans*, Poly$(Q)_{44}$-YFP proteins are sequestered into aggregates in a poly-ubiquitin-dependent process. We cannot formally rule out that the observed decrease in aggregates in *dop-1* mutants is not due to an increase in aggregate clearance in those mutants. We have therefore addressed this issue in several different assays that probe proteostasis. For example, we find that heat shock proteins are elevated in *dop-1* mutants. As pointed out by reviewer 2, this is indicative of impaired proteostasis. We find that *dop-1* mutants, as well as mutants for the different xenobiotic detoxification genes, are more sensitive to heat shock stress (they are more likely to die after heat shock), which is an additional hallmark of impaired proteostasis. Also pointed out by reviewer 2, the decrease in Ub^{G76V}-GFP turnover in *dop-1* mutants could be due to an increase in the amount of proteins that are impaired for proper folding, as this increase in unfolded protein burden would likely tax the capacity of the UPS (and other proteostasis mechanisms). The overtaxed capacity of the UPS should show up as a reduction in the rate of a sensitive reporter for the UPS like the Ub^{G76V}-GFP protein. Indeed, we directly tested this possibility by examining the reporter protein in mutants with impaired protein folding capacity: heat shock mutants. We found that Ub^{G76V}-GFP is stabilized in heat shock mutants, consistent with Ub^{G76V}-GFP turnover being sensitive to proteostasis. The lab of Thorsten Hoppe has made similar observations with these reporters (Segref et al, 2011). We have added these additional lines of evidence concerning impaired proteostasis to multiple figures throughout the paper.

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2nd Editorial Decision 12 April 2016

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the three referees and their comments are provided below. As you can see the referees appreciate the introduced changes and support publication here.

There are just a few issues to sort out. Referee #3 has a few minor comments that I would like to ask you to deal with in a revised version.

REFEREE REPORTS

Referee #1:

I am happy with the revisions to the manuscript. I am supportive of publication.

Referee #2:

The authors have sufficiently addressed my major concerns from the original submission with additional experiments and/or altered discussion of their data. The manuscript is improved from the original submission and in my opinion is suitable for publication in EMBO J.

Referee #3:

The authors should be commended for effort to addressed all the reviewers comments and specifically for the ability to except alternative interpretation of their data and follow it experimentally. The paper is well written and I believe will be of high interest to the readers of EMBOJ.

Minor comments:
(1) In the introduction (page 3) second paragraph it is stated "HSP, which act as chaperones". Not all HSP are chaperones and not all chaperones are heat induced it is therefore best to modify the statement to "some of which are chaperones".

(2) In the slow killing exp the authors used FUdR. FUdR can modulate proteostasis and so should be

avoided when examining changes in proteostasis capacity. Because the authors were looking at PA14 toxicity I think this exp does not need to be repeated. But the use of FUdR in Fig 4C should be noted in the legend.

(3) In Fig. 1 B, 1K 2H and 3G the * for statistic significance are not aligned with the data points.

2nd Revision - authors' response 14 April 2016

Reviewer #3 requested that we alter the phrase “HSP, which act as chaperones” to “HSP, some of which act as chaperones” on page 3 of the manuscript. We have made the requested change and uploaded the corrected manuscript.

Reviewer #3 requested that the use of FUdR in the experiments for Figure 4C be indicated in the figure legend. We have made the requested change in the corrected manuscript.

Reviewer #3 was concerned that asterisk to indicate statistical significance was not aligned directly over the data points in Figure 1B, 1K, 2H, and 3G. We have made the requested changes and uploaded the corrected figures.

The revised manuscript referred to two Excel spreadsheets of supporting data from our RNA-seq analysis as “Supplemental Data 1” and “Supplemental Data 2.” We have revised the manuscript to refer to these items as “Table EV1” and “Table EV2,” respectively.

We have included a single PDF containing all original uncropped blots used in Figure 2. This can be added as a supplementary source data file.

THE EMBO JOURNAL

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Corresponding Author Name: Christopher Rongo
Manusript Number: EMBO J 92524R1

Reporting Checklist For Life Sciences Articles

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript (see link list at top right).

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars only for independent experiments and sample sizes where the application of statistical tests is warranted (error bars should not be shown for technical replicates)
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation (see link list at top right).

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Page 20
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Pages 27-32
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA: No animals were excluded.
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Page 20
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Pages 22-23
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition' (see link list at top right). Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GSE80807
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